Chemical Methods for the Simultaneous Quantitation of Metabolites and Proteins from Single Cells

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Supporting Information

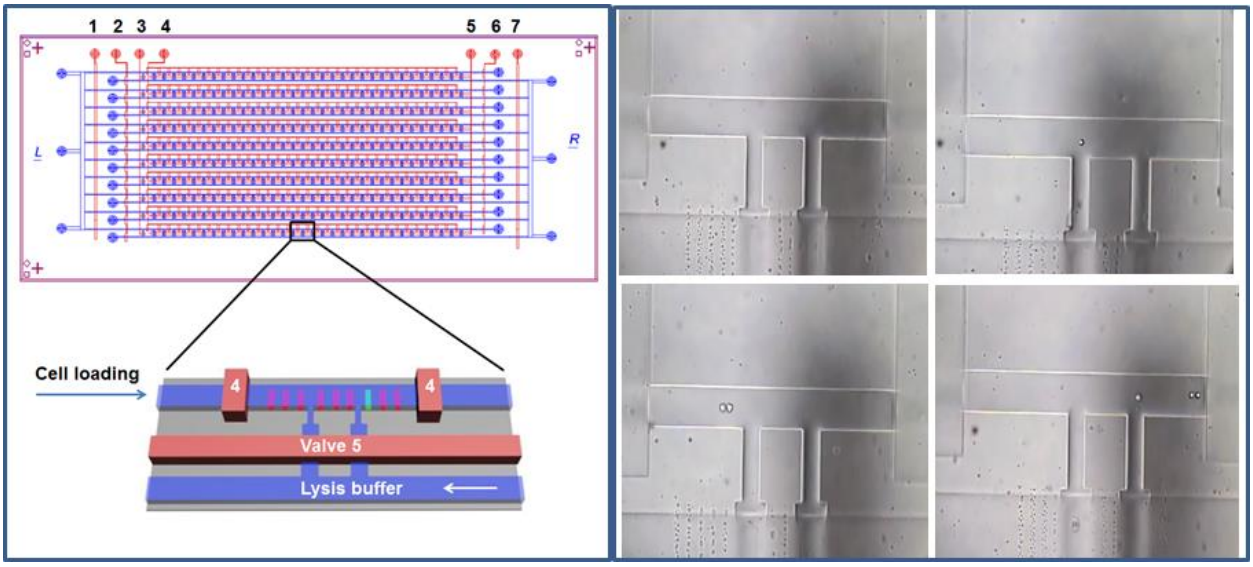

Figure S1. (left) The design of the two-layered SCBC device. The annotations of the valves are labeled on the top. **(right)** The microscopy image of individual cell chambers showing 0, 1, 2 and 3 cells trapped in a chamber.

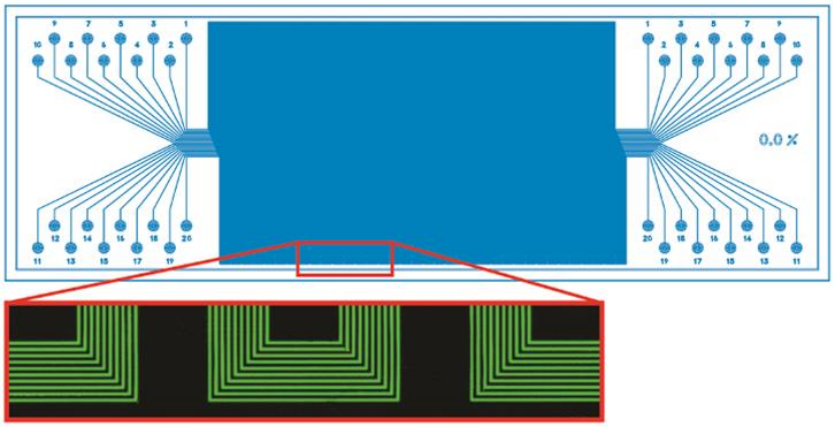

Figure S2. The layout of the DNA barcode patterning. The zoom-in picture shows a typical validation result of patterned DNA through Cy3-labeled complementary DNA hybridization.

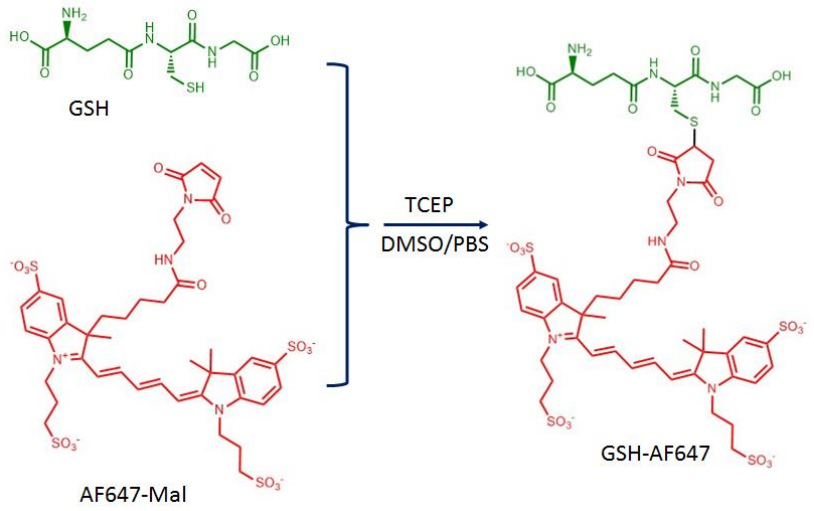

Figure S3. Synthetic scheme of GSH-AF647. The thiol group on the GSH molecule reacts with AF647-maleimide to afford the product. Modification on other sites such as the amine group and the carboxyl groups interfered with antibody binding.

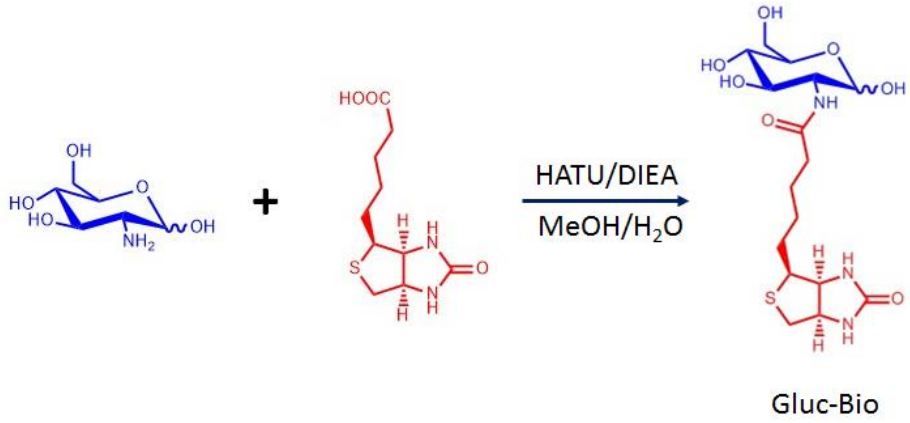

Figure S4. Synthetic scheme of Gluc-Bio. Biotin was linked to glucosamine through HATU coupling. The product is a mixture of α-/β- isomers.

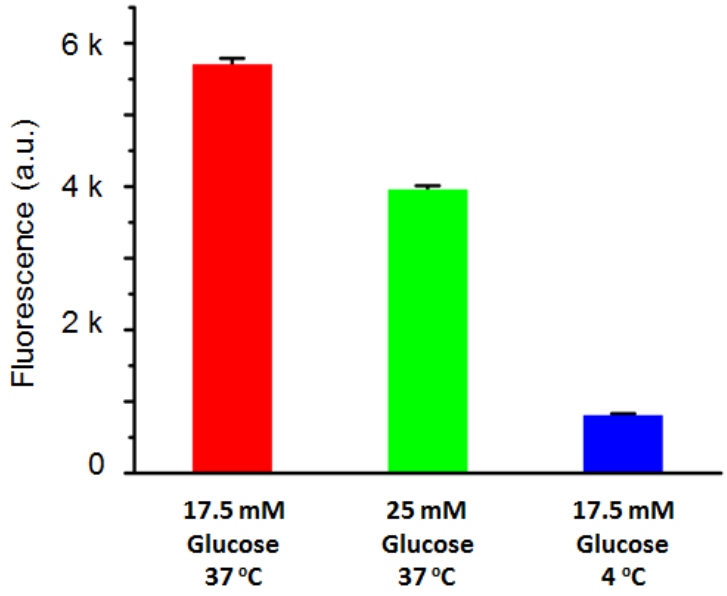

Figure S5. The effect of high glucose concentration and low temperature on the uptake rate of Gluc-Bio. Results prove that the uptake of Gluc-Bio is through the same mechanism as that of glucose.

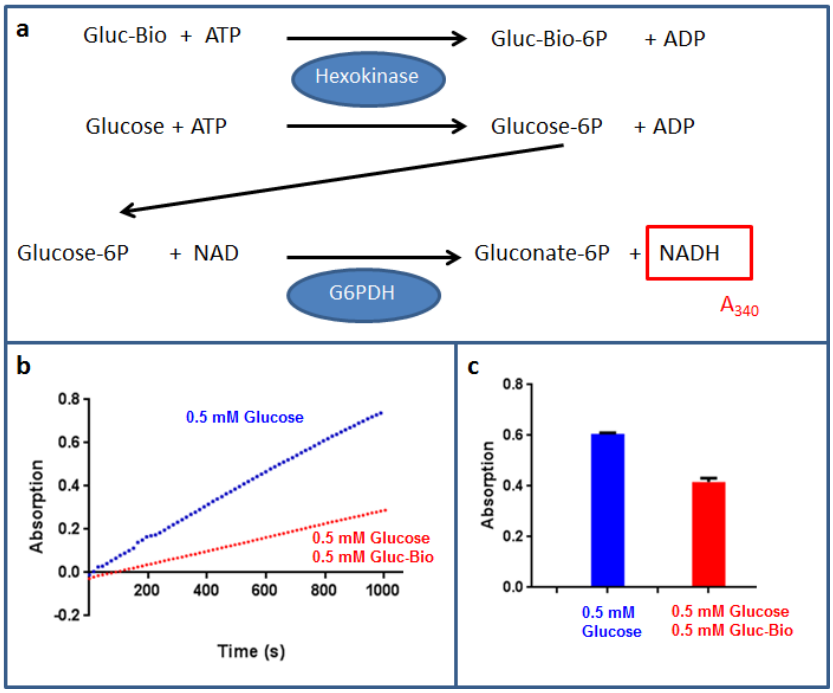

Figure S6. (a) The coupled enzyme reactions allow the assay of hexokinase activities through measuring the absorption of NADH. **(b)** The generation of NADH is much slower with the existence of Gluc-Bio. **(c)** The final amount of NADH is decreased with Gluc-Bio addition.

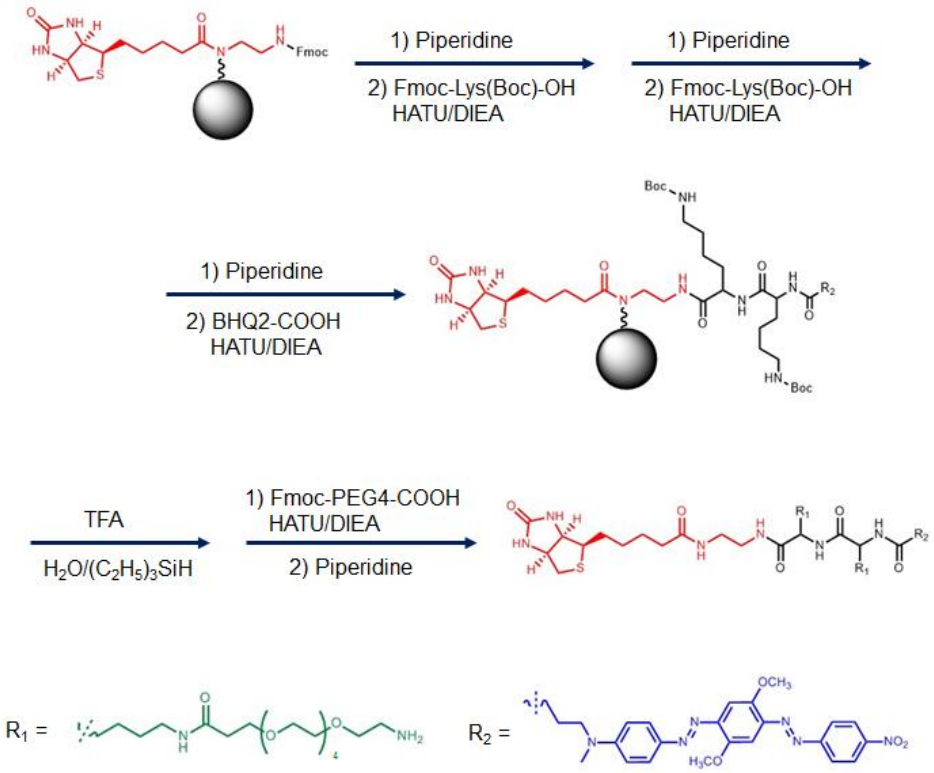

Figure S7. Synthetic scheme of Biotin-BHQ2. The Biotin-Lys-Lys-BHQ2 backbone was prepared through solid state peptide synthesis. The PEG motif was attached to the lysine side chain in solution. The PEG part is essential for enhancing the solubility of Biotin-BHQ2 and minimizing non-specific bindings.

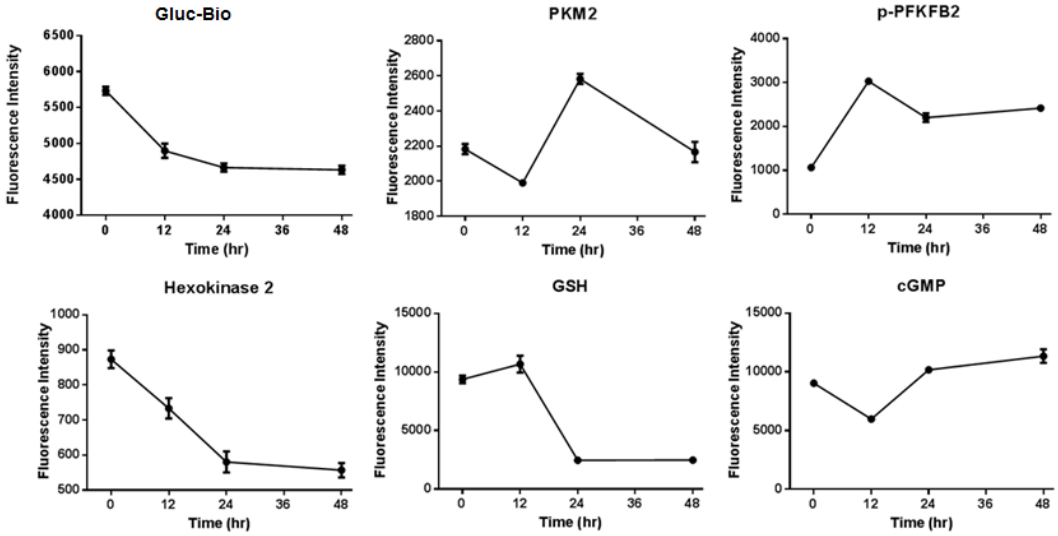

Figure S8. Kinetic immunofluorescence assay results for selective enzymes and metabolites from the GBM39 cells treated with 1 μM of erlotinib. The consistent kinetics of Gluc-Bio uptake and hexokinase 2 confirms that Gluo-Bio is a substrate for hexokinase.

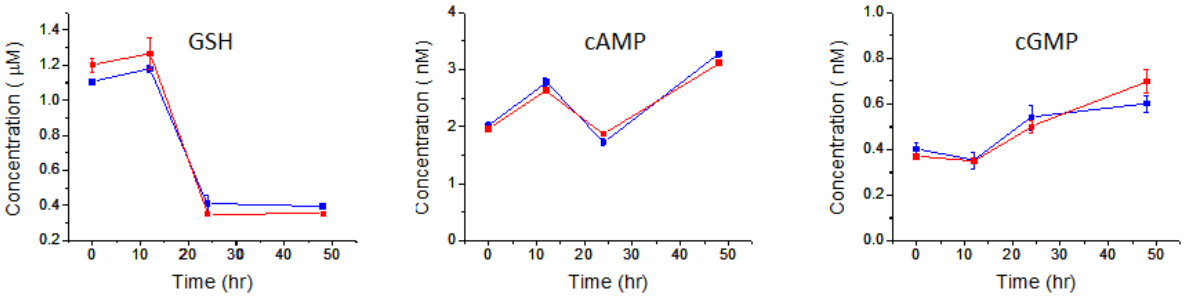

Figure S9. Comparison of metabolite concentrations generated from the immunofluorescence assay used in this work (red traces) and from commercially available assay kits (blue traces. Glutathione Assay Kit, Cayman Chemical #703002; Cyclic AMP XP Assay Kit, Cell Signaling #4339; Cyclic GMP XP Assay Kit, Cell Signaling #4360). These assays utilized the same GBM39 cells and treatments as were performed Figure S8.

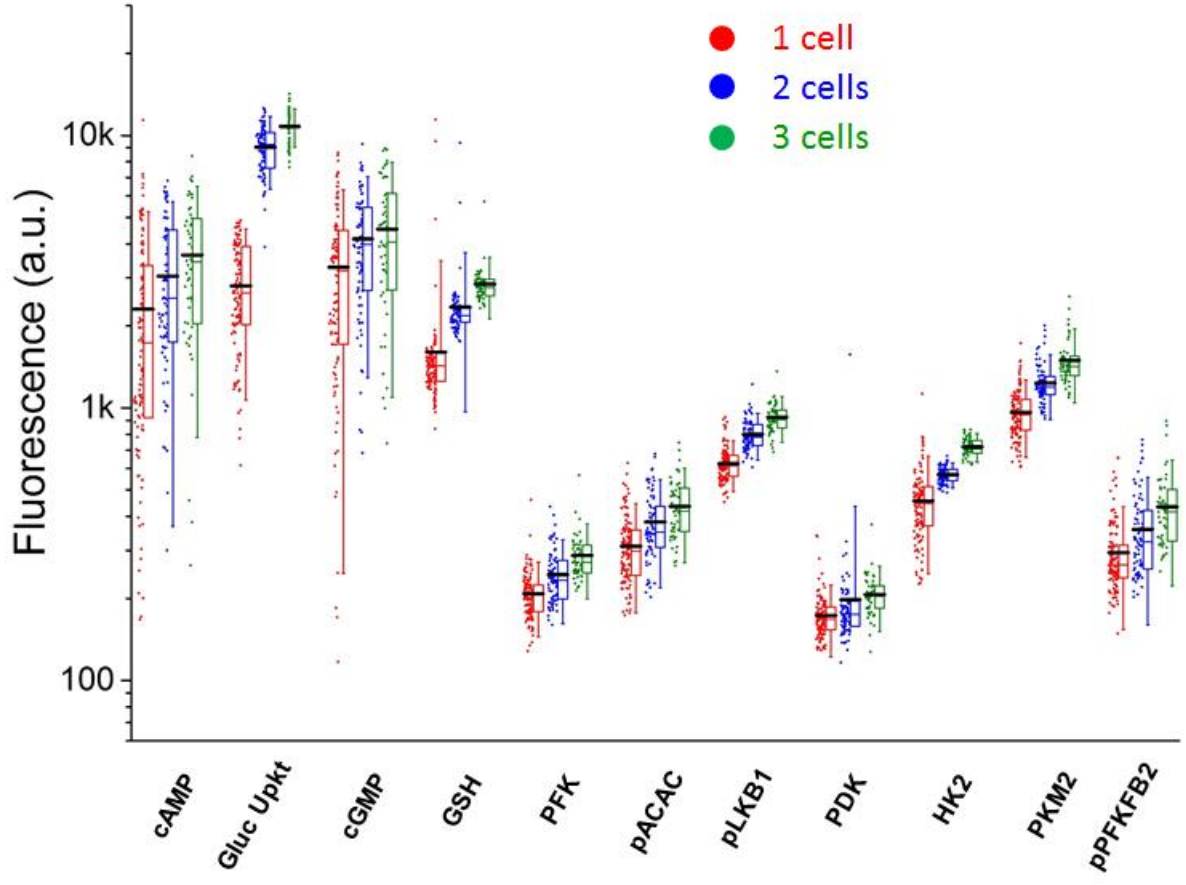

Figure S10. Comparison of signals from 1, 2 and 3 cells generated from the control GBM39 SCBC data. The increasing signal with increased cell number demonstrates the validity of the SCBC assay.

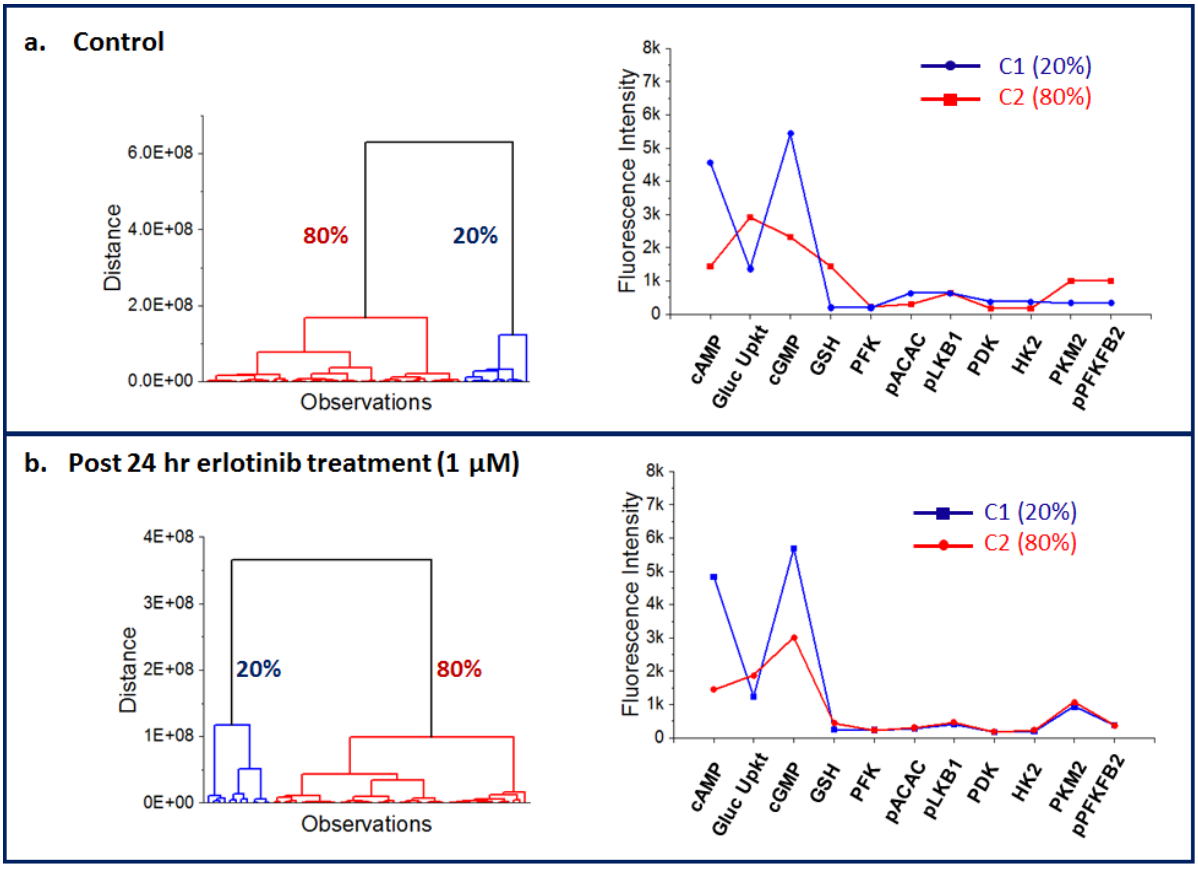

Figure S11. Unsupervised clustering of SCBC data collected from GBM39 neurosphere tumor models before **(a)** and following treatment with an EGFR inhibitor **(b)**. The right panels show the feature of centroids for each population.

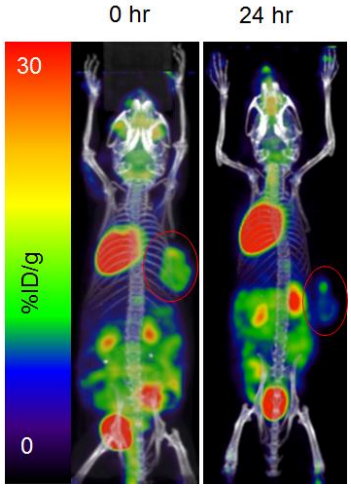

Figure S12. Representative ^{18}F-FDG PET/CT images of GBM39 subcutaneous tumor bearing mice at 0 or 24 hours post treatment with erlotinib (50m/kg, *n*=4 mice) for 24 hours.

Table S1. DNA sequences used in this work.

Name	DNA Sequence
B	5'-NH2-C6-AAA AAA AAA AGC CTC ATT GAA TCA TGC CTA -3'
B'	5'-NH2-C6-AAA AAA AAA ATA GGC ATG ATT CAA TGA GGC -3'
C	5'-NH2-C6-AAA AAA AAA AGC ACT CGT CTA CTA TCG CTA -3'
C'	5' -NH2-C6-AAA AAA AAA ATA GCG ATA GTA GAC GAG TGC -3'
D	5'-NH2-C6-AAA AAA AAA AAT GGT CGA GAT GTC AGA GTA -3'
D'	5'-NH2-C6-AAA AAA AAA ATA CTC TGA CAT CTC GAC CAT -3'
E	5'-NH2-C6-AAA AAA AAA AAT GTG AAG TGG CAG TAT CTA -3'
E'	5' -NH2-C6-AAA AAA AAA ATA GAT ACT GCC ACT TCA CAT -3'
F	5-NH2-C6-AAA AAA AAA AAT CAG GTA AGG TTC ACG GTA -3'
F'	5' -NH2-C6-AAA AAA AAA ATA CCG TGA ACC TTA CCT GAT -3'
H	5'-NH2-C6-AAA AAA AAA AAT TGA CCA AAC TGC GGT GCG-3'
H'	5' -NH2-C6-AAA AAA AAA ACG CAC CGC AGT TTG GTC AAT-3'
I	5'-NH2-C6-AAA AAA AAA ATG CCC TAT TGT TGC GTC GGA-3'
I'	5' -NH2-C6-AAA AAA AAA ATC CGA CGC AAC AAT AGG GCA-3'
K	5'-NH2-C6-AAA AAA AAA ATA ATC TAA TTC TGG TCG CGG-3'
K'	5' -NH2-C6-AAA AAA AAA ACC GCG ACC AGA ATT AGA TTA-3'
L	5'-NH2-C6-AAA AAA AAA AGT GAT TAA GTC TGC TTC GGC-3'
L'	5' -NH2-C6-AAA AAA AAA AGC CGA AGC AGA CTT AAT CAC-3'
N	5' -NH2-C6-AAA AAA AAA AGT CCT CGC TTC GTC TAT GAG-3'
N'	5'-NH2-C6-AAA AAA AAA ACT CAT AGA CGA AGC GAG GAC-3'
O	5' -NH2-C6-AAA AAA AAA ACT TCG TGG CTA GTC TGT GAC -3'
O'	5' -NH2-C6-AAA AAA AAA AGT CAC AGA CTA GCC ACG AAG-3'

Table S2. List of capture antibodies and their manufactures used in this work. The corresponding DNA names for DEAL conjugation is also provided.

DNA	Capture Antibody Name	Manufacture
B'	cAMP Antibody, Rabbit Polyclonal	GenScript, A00614
C'	Streptavidin Antibody, Mouse Monoclonal	GeneTex, GTX10020
D'	cGMP Antibody, Rabbit Polyclonal	GenScript, A00615
E'	Glutathione Antibody, Mouse Monoclonal	GeneTex, GTX16200
F'	Human/Mouse/Rat PFKM Antibody, Sheep Polyclonal	R&D Systems, AF7687
H'	Human/Mouse/Rat Acetyl-CoA Carboxylase Antibody, Sheep Polyclonal	R&D Systems, AF6898
I'	Human LKB1 Antibody, Sheep Polyclonal	R&D Systems, AF8055
K'	Anti-PDK1 Antibody, Mouse Monoclonal	Abcam, ab110335
L'	Hexokinase II Antibody, Rabbit Monoclonal	Cell Signaling, 2867
N'	Human/Mouse/Rat PKM2 Antibody, Sheep Polyclonal	R&D Systems, AF7244
O'	PFKFB2 Antibody, Rabbit Monoclonal	Cell Signaling, 13029

Table S3. List of detection antibodies used in this work.

Catalog#	Detection Antibody Name	Manufacture
ab8326	Anti-HRP Antibody, Mouse Monoclonal	Abcam
12746	PFKP Antibody, Rabbit Monoclonal	Cell Signaling
11818	Phospho-Acetyl-CoA Carboxylase (Ser79) Antibody, Rabbit Monoclonal	Cell Signaling
3482	Phospho-LKB1 (Ser428) Antibody, Rabbit Monoclonal	Cell Signaling
3820	PDHK1 Antibody, Rabbit Monoclonal	Cell Signaling
MABN702	Anti-HK2 Antibody, Mouse Monoclonal	EMD Millipore
4053	PKM2 Antibody, Rabbit Monoclonal	Cell Signaling
13064	Phospho-PFKFB2 (Ser483) Antibody, Rabbit Monoclonal	Cell Signaling

Chemicals and Reagents

Reduced L-glutathione (GSH, 98%), tris(2-carboxyethyl)phosphine hydrochloride (TCEP, 98%), biotin (99%), glucosamine hydrochloride (99%), 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU, 97%), N,N-diisopropylethylamine (DIEA, 99%), citric acid (99.5%), sodium azide (99.5%), N-methyl-2-pyrrolidone (NMP, 99%), trifluoroacetic acid (TFA, 99%), triethylsilane (99%), piperidine (99.5%) and glucose assay kit (GAHK-20) were purchased from Sigma Aldrich (St. Louis, MO). Phosphate buffered saline was purchased from IrvineScientific (Santa Ana, CA). Alexa Fluor 647 C2-Maleimide (AF647-Mal) and Alexa Fluor 647 NHS ester (AF647-NHS) were purchased from Life Technologies (Grand Island, NY). cAMP-HRP and cGMP-HRP conjugates were purchased from GeneScript (Piscataway, NJ). Methanol, dichloromethane, ethyl ether, deionized water and HPLC-grade acetonitrile were purchased from VWR (Radnor, PA). Succinimidyl 4-hydrazinonicotinate acetone hydrazine (S-HyNic), succinimidyl 4-formylbenzoate (S-4FB) and anhydrous N,N-dimtheylformamide (DMF) were purchased from Solulink (San Diego, CA). Biotin NovaTag^{TM} resin, Fmoc-Lys(Boc)-OH, Fmoc-NH-(PEG)4-COOH were purchased from Millipore (Temecula, CA). BHQ-2 carboxylic acid was obtained from Biosearch Technologies (Petaluma, CA)

Synthetic procedures

Glutathione-AlexaFluor 647 (GSH-AF647) The synthetic scheme of GSH-AF647 is shown in **Fig S3**. 100 uL of 10 mM reduced glutathione in 1X PBS was mixed with 10 uL of 100 mM TCEP in 1X PBS and the solution was shaked at room temperature for 1 hr. Subsequently, 100 uL of AF647-Mal (10 mM, in DMSO) was added to the solution and the mixture was shaked in dark at room temperature for 2 hours. The crude was separated using reverse-phase HPLC (C18 column, A:H_2O, B:CH_3CN, 100%A to 30%A). The fractions were collected and lyophilized. The product was characterized by mass spec (MOLDI-TOF, $[M+H+K]^{2+}$ calculated: 656.635, observed: 656.433).

Glucose-biotin (Gluc-Bio) The synthesis of Gluc-Bio is shown in **Fig S4**. In a typical synthesis, 270 mg of biotin was dissolved in 30 mL of methanol and 2 mL of N,N-diisopropylethylamine. 380 mg of HATU was added into the solution and thoroughly mixed. In another flask, 215 mg of 2-glucosamine hydrochloride was suspended in 5 mL deionized water. This suspension was mixed with the biotin/HATU solution and stirred at room temperature for 3 hr. After removing the solvent under vacuum, the crude was mixed with 5 mL of water. The resulting mixture was filtered and the supernatant was purified using HPLC (C18 column, A:H_2O, B:CH_3CN, 100%A to 50%A). The product was characterized by mass spec (MOLDI-TOF, $[M+Na]^+$ calculated: 428.147, observed: 428.289).

Biotin-BHQ2 The synthesis of Biotin-BHQ2 was based on the solid state peptide synthesis technique. The synthetic scheme is shown in **Fig S7**. 100 mg of Biotin-NovaTag^{TM} resin was suspended in 5 mL of NMP for 2 hr, and then treated with piperidine (5 mL and 10 min, repeat 3

times) to perform Fmoc-deprotection. The resin was then washed with 5 mL of NMP (20% in NMP) and resuspended in 1 mL of NMP. HATU (0.2 M in NMP, 2 mL), Fmoc-Lys(Boc)-OH (0.2 M in NMP, 2 mL) and DIEA (0.5 mL) were added and the mixture was incubated at room temperature for 1 hour. The solvent was then removed and the resin was washed with NMP (5 mL) for 3 times. Similar synthetic steps were carried out twice to attach another Fmoc-Lys(Boc)-OH and a BHQ2-COOH on to the resin, respectively. After synthesis, the resin was sequentially washed with NMP, methanol and dichloromethane and dried in air. To perform peptide cleavage, the resin was stirred in 5 mL of TFA with 2.5% water and 2.5% triethylsilane at room temperature for 2 hr. The mixture was filtered to remove the resin. The supernatant was mixed with 10 mL of ethyl ether and evaporated in air flow to afford a dark blue solid. This solid was then dissolved in 10 mL of acetonitrile/methanol (1/1) and then mixed with HATU (0.2 M in acetonitrile, 2 mL), Fmoc-NH-PEG4-COOH (0.4 M in acetonitrile, 1 mL) and DIEA (0.5 mL). The solution was stirred at room temperature for 3 hr. Subsequently, 10 mL of piperidine was added into the solution and mixed for 1 hr. The solvent was then removed under reduced pressure, resulting in a black solid. The solid was resuspended in 10 mL of water and then filtered. The supernatant was purified through HPLC to afford the product. (MOLDI-TOF, $[M+H]^+$ calculated: 1612.853, observed: 1613.186).

Antibody AF 647 conjugation

Commercial antibodies were purified with protein A/G resins (Pierce) following manufacture's protocols. The purified antibodies were buffer exchanged into pH 7.4 PBS buffer using Zeba protein desalting spin columns (Pierce). Purified detection antibodies were reacted with AF647-NHS following manufacture's protocol (Life technologies). The degree of labeling was confirmed by absorption spectra. The labeled antibodies were stored in pH 7.4 PBS buffer with 0.02% NaN_3 as preservatives. The list of capture antibodies and their manufactures are shown in Table S3.

DNA-encoded antibody library

The DNA-encoded antibody library method was based on the literature (R. Bailey, et al., *J. Am. Chem. Soc.,* **2007**, *129*, 1959-1967). 1 mg of S-HyNic and 1 mg of S-4FB were dissolved in 172 uL and 50 uL of anhydrous DMF respectively. 100 ug of the purified antibody was reconstituted in PBS buffer at 1 mg/mL and 5 uL of the S-HyNic solution was added. In another vial, 50 uL of an ssDNA oligo solution (200 uM in PBS) was mixed with 12.5 uL of DMF, and 5 uL of the S-4FB solution was added. The antibody and the DNA solutions were incubated at room temperature for 2 hr. The excess of the S-HyNic and S-4FB were removed through buffer exchanging into a pH 6.0 citrate buffer using Zeba spin columns. The antibody and DNA solutions were then combined and incubated at room temperature for 2 hrs then at 4 oC overnight. The DNA-Antibody conjugate was purified through FPLC (GE, Pharmacia Superdex 200 gel filtration column) and concentrated by centrifugal filter (Millipore, Amicon Ultra-4, 10kD). The list of antibodies used in this work is shown in Table S2.

DNA patterning on glass slides

DNA barcode patterns are prepared using previously reported methods (J. Yu, et al., *Annu. Rev. Anal. Chem.*, **2014**, *7*, 275-295). First a master mold was prepared using the SU8 2035 photoresist. The mold was patterned with parallel channels at 10 um wide and 40 um high. A

Sylgard 184 (A:B=7:1) polymer mix was poured onto the mold, degassed under vacuum and then cured at 80 ^{o}C for 2 hours. The cured PDMS was released from the mold and bonded onto a poly-L-lysine (PLL) coated glass slide (Thermo Scientific) to form enclosed channels. The number of analytes determines the number of the channels (11 channels in this study). The channels were flushed with a 0.1% PLL solution (Sigma Aldrich) and blown dry by air. Meanwhile, 5'-amine modified ssDNA was dissolved in a DMSO/H_2O (2:3) solution at 300 uM and mixed with a 2 mM PBS solution of BS3 linker (Thermo Fisher) at 1:1 ratio (v/v). A library of freshly prepared DNA solutions were flown into different channels and the assembly was incubated at room temperature for 2 hours. The glass slide was then separated from the PDMS slab and washed with 0.02% SDS solution and water. Each patterned slide was validated using a solution of Cy3-labeled complementary DNA mixtures at one edge, and the fluorescence intensity of the barcodes were measured with an Axon GenPix 4400A scanner. The sequences of 5'-amine modified ssDNA are listed in **Table S1**, and the layout of the DNA patterning mold is shown in **Fig S2**.

Preparation of single cell suspensions

GBM 39 neurospheres were collected via centrifugation at 450 g for 5 min and the media was removed. The cells were then treated with 0.05% Tripsin/EDTA (Life Technologies) for 5 min at room temperature and the original media was added back to the pellet. The suspension was then centrifuged again and the supernatant was discarded. The cells are now disassociated as single cells and ready for tests.

For SCBC measurements, the cells were resuspended in warm media with 10 ug/mL of Gluc-Bio at 6X10^5 cells/mL. The cells were incubated at 37 ^{o}C for 20 min and washed with cold PBS for three times. After washing, the cells were resuspended in serum-free, biotin-free media at a concentration of 1 M cells/mL and ready for loading.

Cell culture and drug treatment

GBM39 primary neurospheres were provided by Prof. C. David James (UCSF, San Francisco, U.S.A.) and cultured in Dulbecco's Modified Eagle Media Nutrient Mix F-12 (DMEM/F12, Invitrogen) supplemented with B27 (Invitrogen), Glutamax (Invitrogen), Heparin (1μg/mL), Epidermal Growth Factor (EGF, 20ng/mL, Sigma), Fibroblast Growth Factor (FGF, 20ng/mL, Sigma) and 100 U/mL of penicillin and streptomycin (Gibco) in a humidified 5% CO_2 (v/v) incubator, at 37 ^{o}C. For the drug treatment, 1 million cells were suspended in 10 mL of media and was cultured for several days to form neurosphere. Subsequently, the medium was changed to 10 mL of new media containing 1 μM erlotinib (ChemieTek). The cells were then treated for designated periods of time and processed for tests.

Gluc-Bio / FDG kinetic measurements

Aforementioned drug treated GBM 39 cells were dissociated into single cells and re-suspended in pre-warmed glucose-free, serum-free media (DMEM, Invitrogen) at a concentration of 10^5 cells/mL (for FDG) or 2×10^5 cells/mL (for Gluc-Bio). Concentrated aqueous solution of Gluc-Bio or FDG was added to the cell suspension (final concentration: 10 μg/mL of Gluc-Bio, 4 uCi/mL of FDG). The cells were incubated at 37 ^{o}C for 10 min (Gluc-Bio) or 1 hr (FDG). At the end of

incubation, the cells were collected via centrifugation and washed three times with cold PBS buffer (2X original media volume). The resulting cells were either lysed for analyzing the Gluc-Bio amount or re-suspended in PBS to measure the FDG content with a γ counter (1480 Wizard 3; Perkin Elmer) .

Fabrication of single cell barcode chip (SCBC) devices

The SCBC devices consist of DNA barcode microarray glass slides and PDMS slabs that contain microfluidic circuits. The DNA barcode slides were prepared through microchannel-guided flow patterning method **Fig S2**. The PDMS slabs were fabricated using a two-layer soft lithography approach. The flow layer was fabricated by spin-casting Sylgard 184 (A:B=20:1) polymer mix onto a SPR 220 positive photoresist mold (16 um in height) at 2000 rpm for 1 min. The control layer was molded from a SU8 2025 negative photoresist master (40 um in height) using Sylgard 184 (A:B=7:1) polymer mix. These two parts were cured at 80 ^{o}C for 20 min, and the control layer was aligned onto the flow layer. The two-layer device was thermally treated at 80 ^{o}C (1.5 hr) and then released from the flow layer mold. After drilling the inlet/outlet holes from the PDMS, the slab was aligned onto the DNA barcode glass slides and treated at 80 ^{o}C (2 hr) to afford a fully assembled SCBC device. The layout of the device is illustrated in **Fig S1**.

Operation of the SCBC devices

1. **Conversion of the DNA barcodes to capture antibody microarrays.** The control valve 5 (**Fig S1**) was closed to separate the fluidic channels into upper and lower channels. The upper channels can be further compartmentalized into different operational units (microchambers), each of which was equipped with a full set of antibody barcode for cell trapping and analyte detection, while the lower ones were used as lysis buffer reservoir (**Fig S1**). A cocktail of DNA-encoded antibody conjugates in a BSA solution (1.5% in PBS) was flown into the upper channels and incubated at 37 ^{o}C for 1 hr to convert the DNA barcodes into antibody microarrays. The unbound conjugates were then washed off by 1X PBST (Cell Signaling, 0.05% Tween 20) and the channels were then blocked with a BSA (1% in 1X PBST) solution for 1 hr.
2. **Cell loading.** The upper channels were briefly rinsed with cell culture medium to minimize perturbation to the cells. Simultaneously, GBM39 neurospheres were disassociated into single cells and incubated with Gluc-Bio. After washing to remove the excess Gluc-Bio, these single-cells were suspended in serum-free, biotin-free media at a concentration of 10^6 cells/mL. The suspension was loaded into the SCBC device and compartmentalized into 310 isolated microchambers with single cell or defined number of cells in each chamber. The cell loading step results in a random distribution of different numbers of cells in different chambers, the statistical distribution can be adjusted by varying the loading concentration and the flow speed. In our experiment setup, each chip typically has around 100 zero-cell chambers, 100 single cell chambers and 100 chambers with more than 2 cells. This distribution is critical in terms of signal background subtraction and validation. The extra cells were washed away by 1X PBST. The images of each chamber were recorded using a microscope-CCD camera and used for subsequent cell counting.

3. **On-chip cell lysis.** A cell lysis buffer mixture was introduced into the lower channels and the whole device was placed on ice. Valve 5 was opened for 15 min to allow the lysis buffer to diffuse into individual cell chambers under a positive pressure. After closing valve 5, the device was incubated on ice for another 15 min to complete the on-chip cell lysis. The device was then incubated at room temperature with shaking for 2 hr to complete the capture of analytes by the antibody microarrays. After the incubation, the unbound cell lysate was quickly flushed away by 1X PBST.
4. **Applying the detection cocktail**. A cocktail of Alexa Fluor 647-labeled detection antibodies as well as the Biotin-BHQ2 probe were prepared in a BSA solution (1% in 1X PBST) and flown into the device for 60 min to develop the analytes captured into fluorescence signal.
5. **Rinse and fluorescence readout**. The channels were washed with 1X PBST for 30 min. The barcode slide was then peeled off from the PDMS slab, washed sequentially with 1X PBS, 0.5X PBS and Millipore water, dried by a VWR Miniarray microcentrifuge, and scanned by an Axon GenePix 4400A at laser power 80% (635 nm) and 15% (532nm), and at 2.5 um/pixel resolution. Signals from two color channels were collected and digitized by the manufactures' software.

Generation of calibration curves

The standard calibration of metabolites were performed using SCBC devices under the same conditions described above, except that known concentrations of metabolite solutions were loaded into the device instead of the cells. The fluorescence signals from the resulted barcode slides were collected to generate the calibration curves (**Fig 1d**). These standard curves allow converting the relative fluorescence signal into copy number of analytes assayed, enabling absolute quantifications of the metabolites, under the condition that the same concentration of the competitors were employed in the experiments.

Statistical analysis

The SCBC dataset is an $m \times n$ matrix table, each row (***m***) of which represents a specific microchamber address with a defined number of single cells and each column (***n)*** represents the abundance of a specific analyte in those microchambers. Pairwise comparison was performed through Mann-Whitney method. The correlation coefficients were directly calculated from the dataset using Spearman's rank method. Bonferroni corrected p-value was used to define the statistical significance level for the entire panel and only significant ones were shown in the correlation network (**Fig 3a**). Agglomerative hierarchical clustering analysis was carried out by XLSTAT software (Addinsoft) on the single cell dataset. The proximity among single cell readouts was measured by the dissimilarity coefficients of Euclidian distance with Ward's minimum variance method. The calculated dissimilarity coefficients were employed to quantify the functional heterogeneity of the cells.

Cell lysis buffer mixture recipe used in SCBC measurements

400 uL of 10X cell lysis buffer (Cell Signaling), 20 uL of 100X protease/phosphatase inhibitor cocktail (Cell Signaling), 100 uL of 1.5% BSA/PBS, 3.3 uL cAMP-HRP (Genscript, 0.1 mg/mL), 4.4 uL cGMP-HRP (Genscript, 0.1 mg/mL), 1 uL of Alexa Fluor 555-lebeled streptavidin (0.1 mg/mL) and 0.5 uL GSH-AF647 (22.6 uM in PBS)

Validation of the Gluc-Bio uptake

GBM39 single cells were suspended in normal media (17.5 mM glucose) or high glucose media (25 mM glucose) at a concentration of 0.5 M cells/mL. These cells were incubated with 20 ug/mL of Gluc-Bio at 37 ^{o}C or 4 ^{o}C for 20 min. After incubation, cells were washed with cold PBS for three times and then lysed for quantifying the Gluc-Bio. The result is shown in **Fig S5**.

Validation of the Gluc-Bio as a substrate for hexokinase

In order to verify that Gluc-Bio is a substrate for hexokinase, a coupled enzyme kinetics measurement method was employed. Enzymes and substrate solutions from a glucose assay kit (Sigma-Aldrich) were used without purification. **Fig S6a** shows the two coupled enzymatic reactions. First, hexokinase converts glucose into glucose-6-phosphate, which consumes one equivalent of ATP. Then, the glucose-6-phosphate is converted to gluconate-6-phosphate by glucose-6-phosphate dehydrogenase (G6PDH). At the same time, this process converts NAD to NADH, which has a distinct absorption band at 340 nm. By continuously measuring the solution absorption, the kinetics of the first reaction can be indirectly monitored. If Gluc-Bio is a substrate for hexokinase, the product 2'-biotinyl-deoxyglucose-6-phosphate (Gluc-Bio-6P) cannot be further converted by G6PDH and therefore will not increase the NADH amount. This will result in a much slower generation of NADH. **Fig S6b** demonstrates that in the presence of Gluc-Bio, the generation of NADH is much slower than that of glucose alone. This proves that Gluc-Bio can bind to hexokinase and could be a substrate. However, this result cannot distinguish whether Gluc-Bio is a substrate or an inhibitor. In order to clarify this, another experiment was designed to prove that Gluc-Bio is a substrate. In this experiment, the amount of ATP was controlled to be much less than that of glucose. If Gluc-Bio is simply an inhibitor and cannot be converted by hexokinase, it will not consume the ATP pool in the solution, and the final amount of NADH will be the same as that of glucose alone. **FigS6c** shows that with the addition of Gluc-Bio, less NADH was generated. This proves that Gluc-Bio consumes ATP and therefore is a substrate for hexokinase.

PET/CT methods: Mice kept warm under gas anesthesia (2% isoflurane) were injected intraperitoneally with ^{18}F-FDG (20 µCi) and microPET scans were acquired for 10 minutes on the Genisys4 bench-top PET scanner (Sofie Biosciences). CT images were acquired the MicroCAT II CT system (Siemens) PET data are reconstructed into multiple frames using a statistical maximum *a posteriori* probability algorithm (MAP). CT (MicroCAT) images are at low dose 400 µm resolution acquisition with 200 µm voxel size. PET and CT images are co-registered as previously described (Nathanson, D. A.; et al. *J. Exp. Med.* **2014**, *211*, 473-486).

MicroPET data analysis. Following MAP reconstruction, regions of interest (ROI) of were defined, both over tumors and the background, on the reconstructed images. ROI values were determined by normalization to the total radioactivity injected per body weight (%ID/g) to give the standardized uptake value (SUV).